

PATENT  
406060-2130

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE  
APPLICATION FOR LETTERS PATENT

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Title: ANTINEOPLASTIC COCOA EXTRACTS AND  
METHODS FOR MAKING AND USING THE SAME

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ANTINEOPLASTIC COCOA EXTRACTS AND  
METHODS FOR MAKING AND USING THE SAME

FIELD OF THE INVENTION

5 This invention relates to cocoa extracts such as polyphenols preferably polyphenols enriched with procyanidins. This invention also relates to methods for preparing such extracts, as well as to uses for them; for instance, as antineoplastic agents and antioxidants.

10 Documents are cited in this disclosure with a full citation for each appearing in a References section at the end of the specification, preceding the claims. These documents pertain to the field of this invention; and, each document cited herein is hereby incorporated  
15 herein by reference.

BACKGROUND OF THE INVENTION

Polyphenols are an incredibly diverse group of compounds (Ferreira et al., 1992) which widely occur in a variety of plants, some of which enter into the food  
20 chain. In some cases they represent an important class of compounds for the human diet. Although some of the polyphenols are considered to be nonnutrative, interest in these compounds has arisen because of their possible beneficial effects on health. For instance, quercetin (a  
25 flavonoid) has been shown to possess anticarcinogenic activity in experimental animal studies (Deshner et al., 1991 and Kato et al., 1983). (+)-Catechin and (-)-epicatechin (flavan-3-ols) have been shown to inhibit Leukemia virus reverse transcriptase activity (Chu et  
30 al., 1992). Nobotanin (an oligomeric hydrolyzable tannin) has also been shown to possess anti-tumor activity (Okuda et al., 1992). Statistical reports have also shown that stomach cancer mortality is significantly lower in the tea producing districts of Japan.  
35 Epigallocatechin gallate has been reported to be the pharmacologically active material in green tea that

inhibits mouse skin tumors (Okuda et al., 1992). Ellagic acid has also been shown to possess anticarcinogen activity in various animal tumor models (Bukharta et al., 1992). Lastly, proanthocyanidin oligomers have been  
5 patented by the Kikkoman Corporation for use as antimutagens. Indeed, the area of phenolic compounds in foods and their modulation of tumor development in experimental animal models has been recently presented at the 202nd National Meeting of The American Chemical  
10 Society (Ho et al., 1992; Huang et al., 1992).

However, none of these reports teaches or suggests cocoa extracts, any methods for preparing such extracts, or, any uses as antineoplastic agents for cocoa extracts.

15 Since unfermented cocoa beans contain substantial levels of polyphenols, the present inventors considered it possible that similar activities of and uses for cocoa extracts, e.g., compounds within cocoa, could be revealed by extracting such compounds from cocoa  
20 and screening the extracts for activity. The National Cancer Institute has screened various *Theobroma* and *Herrania* species for anti-cancer activity as part of their massive natural product selection program. Low levels of activity were reported in some extracts of  
25 cocoa tissues, and the work was not pursued. Thus, in the antineoplastic or anti-cancer art, cocoa and its extracts were not deemed to be useful; i.e., the teachings in the antineoplastic or anti-cancer art lead the skilled artisan away from employing cocoa and its  
30 extracts as cancer therapy. Since a number of analytical procedures were developed to study the contributions of cocoa polyphenols to flavor development (Clapperton et al., 1992), the present inventors decided to apply analogous methods to prepare samples for anti-cancer  
35 screening, contrary to the knowledge in the

antineoplastic or anti-cancer art. Surprisingly, and contrary to the knowledge in the art, e.g., the National Cancer Institute screening, the present inventors discovered that cocoa polyphenol extracts which contain  
5 procyanidins, have significant utility as anti-cancer or antineoplastic agents. Additionally, the inventors demonstrate that cocoa extracts containing procyanidins have utility as antioxidants.

OBJECTS AND SUMMARY OF THE INVENTION

10 It is an object of the present invention to provide a method for producing cocoa extract.

It is another object of the invention to provide a cocoa extract.

15 It is another object of the invention to provide an antioxidant composition.

It is another object of the invention to demonstrate inhibition of DNA topoisomerase II enzyme activity.

20 It is yet another object of the present invention to provide a method for treating tumors or cancer.

It is still another object of the invention to provide an anti-cancer, anti-tumor or antineoplastic composition.

25 It is a further object of the invention to provide a method for making an anti-cancer, anti-tumor or antineoplastic composition.

And, it is an object of the invention to provide a kit for use in treating tumors or cancer.

30 It has been surprisingly discovered that cocoa extract has anti-tumor, anti-cancer or antineoplastic activity; or, is an antioxidant composition or, inhibits DNA topoisomerase II enzyme activity. Accordingly, the present invention provides a substantially pure cocoa  
35 extract. The extract preferably comprises polyphenol(s)

such as polyphenol(s) enriched with cocoa procyanidin(s),  
such as polyphenols of at least one cocoa procyanidin  
selected from (-) epicatechin, procyanidin B-2,  
procyanidin oligomers 2 through 12, preferably 2 through  
5 5 or 4 through 12, procyanidin B-5, procyanidin A-2 and  
procyanidin C-1. The present invention also provides an  
anti-tumor, anti-cancer or antineoplastic or antioxidant  
or DNA topoisomerase II inhibitor composition comprising  
a substantially pure cocoa extract or synthetic cocoa  
10 polyphenol(s) such as polyphenol(s) enriched with  
procyanidin(s) and a suitable carrier. The extract  
preferably comprises cocoa procyanidin(s). The cocoa  
extract is preferably obtained by a process comprising  
reducing cocoa beans to powder, defatting the powder and,  
15 extracting active compound(s) from the powder.

The present invention further comprehends a  
method for treating a patient in need of treatment with  
an anti-tumor, anti-cancer, or antineoplastic agent or an  
antioxidant or a DNA topoisomerase II inhibitor  
20 comprising administering to the patient a composition  
comprising an effective quantity of a substantially pure  
cocoa extract or synthetic cocoa polyphenol(s) or  
procyanidin(s) and a carrier. The cocoa extract can be  
cocoa procyanidin(s); and, is preferably obtained by  
25 reducing cocoa beans to powder, defatting the powder and,  
extracting active compound(s) from the powder.

Additionally, the present invention provides a  
kit for treating a patient in need of treatment with an  
anti-tumor, anti-cancer, or antineoplastic agent or  
30 antioxidant or DNA topoisomerase II inhibitor comprising  
a substantially pure cocoa extract or synthetic cocoa  
polyphenol(s) or procyanidin(s) and a suitable carrier  
for admixture with the extract or synthetic polyphenol(s)  
or procyanidin(s).

These and other objects and embodiments are disclosed or will be obvious from the following Detailed Description.

BRIEF DESCRIPTION OF THE DRAWINGS

5           The following Detailed Description will be better understood by reference to the accompanying drawings wherein:

Fig. 1 shows a representative gel permeation chromatogram from the fractionation of crude cocoa  
10 procyanidins;

Fig. 2A shows a representative reverse-phase HPLC chromatogram showing the separation (elution profile) of cocoa procyanidins extracted from unfermented cocoa;

15           Fig. 2B shows a representative normal phase HPLC separation of cocoa procyanidins extracted from unfermented cocoa;

Fig. 3 shows several representative procyanidin structures;

20           Figs. 4A-4E show representative HPLC chromatograms of five fractions employed in screening for anti-cancer or antineoplastic activity;

Fig. 5 and 6A-6D show the dose-response relationship between cocoa extracts and cancer cells ACHN  
25 (Fig. 5) and PC-3 (Figs. 6A-6D) (fractional survival vs. dose,  $\mu\text{g/ml}$ ); M&M2 F4/92, M&MA+E U12P1, M&MB+E Y192P1, M&MC+E U12P2, M&MD+E U12P2;

Fig. 7A to 7H show the typical dose response relationships between cocoa procyanidin fractions A, B,  
30 C, D, E, A+B, A+E, and A+D, and the PC-3 cell line (fractional survival vs. dose,  $\mu\text{g/ml}$ ); MM-1A 0212P3, MM-1 B 0162P1, MM-1 C 0122P3, MM-1 D 0122P3, MM-1 E 0292P8, MM-1 A/B 0292P6, MM-1 A/E 0292P6, MM-1 A/D 0292P6;

Fig. 8A to 8H show the typical dose response  
35 relationships between cocoa procyanidin fractions A, B,

C, D, E, A+B, B+E, and D+E and the KB Nasopharyngeal/HeLa cell line (fractional survival vs. dose,  $\mu\text{g/ml}$ );

MM-1A092K3, MM-1 B 0212K5, MM-1 C 0162K3, MM-1 D 0212K5, MM-1 E 0292K5, MM-1 A/B 0292K3, MM-1 B/E 0292K4, MM-1 D/E

5 0292K5;

Figs. 9A to 9H show the typical dose response relationship between cocoa procyanidin fractions A, B, C, D, E, B+D, A+E and D+E and the HCT-116 cell line (fractional survival vs. dose,  $\mu\text{g/ml}$ ); MM-1 C 0192H5, D  
10 0192H5, E 0192H5, MM-1 B&D 0262H2, A/E 0262H3, MM-1 D&E 0262H1;

Figs. 10A to 10H show typical dose response relationships between cocoa procyanidin fractions A, B, C, D, E, B+D, C+D and A+E and the ACHN renal cell line  
15 (fractional survival vs. dose,  $\mu\text{g/ml}$ ); MM-1 A 092A5, MM-1 B 092A5, MM-1 C 0192A7, MM-1 D 0192A7, M&M1 E 0192A7, MM-1 B&D 0302A6, MM-1 C&D 0302A6, MM-1 A&E 0262A6;

Figs. 11A to 11H show typical dose response relationships between cocoa procyanidin fractions A, B,  
20 C, D, E, A+E, B+E and C+E and the A-549 lung cell line (fractional survival vs. dose,  $\mu\text{g/ml}$ ); MM-1 A 019258, MM-1 B 09256, MM-1 C 019259, MM-1 D 019258, MM-1 E 019258, A/E 026254, MM-1 B&E 030255, MM-1 C&E N6255;

Figs. 12A to 12H show typical dose response  
25 relationships between cocoa procyanidin fractions A, B, C, D, E, B+C, C+D and D+E and the SK-5 melanoma cell line (fractional survival vs. dose  $\mu\text{g/ml}$ ); MM-1 A 0212S4, MM-1 B 0212S4, MM-1 C 0212S4, MM-1 D 0212S4, MM-1 E N32S1, MM-1 B&C N32S2, MM-1 C&D N32S3, MM-1 D&E N32S3;

Figs. 13A to 13H show typical dose response  
30 relationships between cocoa procyanidin fractions A, B, C, D, E, B+C, C+E, and D+E and the MCF-7 breast cell line (fractional survival vs. dose,  $\mu\text{g/ml}$ ); MM-1 A N22M4, MM-1 B N22M4, MM-1 C N22M4, MM-1 D N22M3, MM-1 E 0302M2, MM-1  
35 B/C 0302M4, MM-1 C&E N22M3, MM-1 D&E N22M3;

Fig. 14 shows typical dose response relationships for cocoa procyanidin (particularly fraction D) and the CCRF-CEM T-cell leukemia cell line (cells/ml vs. days of growth; open circle is control, darkened circle is 125  $\mu$ g fraction D, open inverted triangle is 250  $\mu$ g fraction D, darkened inverted triangle is 500  $\mu$ g fraction D);

Fig. 15 A shows a comparison of the XTT and Crystal Violet cytotoxicity assays against MCF-7 p168 breast cancer cells treated with fraction D+E (open circle is XTT and darkened circle is Crystal Violet);

Fig. 15 B shows a typical dose response curve obtained from MDA MB231 breast cell line treated with varying levels of crude polyphenols obtained from UIT-1 cocoa genotype (absorbance (540 nm) vs. Days; open circle is control, darkened circle is vehicle, open inverted triangle is 250  $\mu$ g/ml, darkened inverted triangle is 100  $\mu$ g/ml, open square is 10  $\mu$ g/ml; absorbance of 2.0 is maximum of plate reader and may not be necessarily representative of cell number);

Fig. 15 C shows a typical dose response curve obtained from PC-3 prostate cancer cell line treated with varying levels of crude polyphenols obtained from UIT-1 cocoa genotype (absorbance (540nm) vs. Days; open circle is control, darkened circle is vehicle, open inverted triangle is 250  $\mu$ g/ml, darkened inverted triangle is 100  $\mu$ g/ml and open square is 10  $\mu$ g/ml);

Fig. 15 D shows a typical dose-response curve obtained from MCF-7 p168 breast cancer cell line treated with varying levels of crude polyphenols obtained from UIT-1 cocoa genotype (absorbance (540 nm) vs. Days; open circle is control, darkened circle is vehicle, open inverted triangle is 250  $\mu$ g/ml, darkened inverted triangle is 100  $\mu$ g/ml, open square is 10  $\mu$ g/ml, darkened square is 1  $\mu$ g/ml; absorbance of 2.0 is maximum of plate



reader and may not be necessarily representative of cell number);

Fig. 15 E shows a typical dose response curve obtained from Hela cervical cancer cell line treated with varying levels of crude polyphenols obtained from UIT-1 cocoa genotype (absorbance (540 nm) vs. Days; open circle is control, darkened circle is vehicle, open inverted triangle is 250  $\mu\text{g/ml}$ , darkened inverted triangle is 100  $\mu\text{g/ml}$ , open square is 10  $\mu\text{g/ml}$ ; absorbance of 2.0 is maximum of plate reader and may not be necessarily representative of cell number);

Fig. 15 F shows cytotoxic effects against Hela cervical cancer cell line treated with different cocoa polyphenol fractions (absorbance (540nm) vs. Days; open circle is 100  $\mu\text{g/ml}$  fractions A-E, darkened circle is 100  $\mu\text{g/ml}$  fractions A-C, open inverted triangle is 100  $\mu\text{g/ml}$  fractions D&E; absorbance of 2.0 is maximum of plate reader and not representative of cell number);

Fig. 15 G shows cytotoxic effects at 100  $\mu\text{l/ml}$  against SKBR-3 breast cancer cell line treated with different cocoa polyphenol fractions (absorbance (540nm) vs. Days; open circle is fractions A-E, darkened circle is fractions A-C, open inverted triangle is fractions D&E);

Fig. 15 H shows typical dose-response relationships between cocoa procyanidin fraction D+E on Hela cells (absorbance (540nm) vs. Days; open circle is control, darkened circle is 100  $\mu\text{g/ml}$ , open inverted triangle is 75  $\mu\text{g/ml}$ , darkened inverted triangle is 50  $\mu\text{g/ml}$ , open square is 25  $\mu\text{g/ml}$ , darkened square is 10  $\mu\text{g/ml}$ ; absorbance of 2.0 is maximum of plate reader and is not representative of cell number);

Fig. 15 I shows typical dose-response relationship between cocoa procyanidin fraction D+E on SKBR-3 cells (absorbance (540nm) vs. Days; open circle is

control, darkened circle is 100  $\mu\text{g/ml}$ ; open inverted triangle is 75  $\mu\text{g/ml}$ , darkened inverted triangle is 50  $\mu\text{g/ml}$ , open square is 25  $\mu\text{g/ml}$ , darkened square is 10  $\mu\text{g/ml}$ );

5                    Fig. 15 J shows typical dose-response relationships between cocoa procyanidin fraction D+E on Hela cells using the Soft Agar Cloning assay (bar chart; number of colonies vs. control, 1, 10, 50, and 100  $\mu\text{g/ml}$ );

10                   Fig. 15 K shows the growth inhibition of Hela cells when treated with crude polyphenol extracts obtained from eight different cocoa genotypes (% control vs. concentration,  $\mu\text{g/ml}$ ; open circle is C-1, darkened circle is C-2, open inverted triangle is C-3, darkened inverted triangle is C-4, open square is C-5, darkened square is C-6, open triangle is C-7, darkened triangle is C-8; C-1 = UF-12: horti race = Criollo and description is crude extracts of UF-12 (Brazil) cocoa polyphenols (decaffeinated/detheobrominated); C-2 = NA-33: horti  
15                   race = Forastero and description is crude extracts of NA-33 (Brazil) cocoa polyphenols (decaffeinated/detheobrominated); C-3 = EEG-48: horti race = Forastero and description is crude extracts of EEG-48 (Brazil) cocoa polyphenols (decaffeinated/detheobrominated); C-4 =  
20                   unknown: horti race = Forastero and description is crude extracts of unknown (W. African) cocoa polyphenols (decaffeinated/detheobrominated); C-5 = UF-613: horti race = Trinitario and description is crude extracts of UF-613 (Brazil) cocoa polyphenols (decaffeinated/  
25                   detheobrominated); C-6 = ICS-100: horti race = Trinitario and description is crude extracts of ICS-100 (Brazil) cocoa polyphenols (decaffeinated/detheobrominated); C-7 = ICS-139: horti race = Trinitario and description is crude extracts of ICS-139 (Brazil) cocoa polyphenols  
30                   (decaffeinated/detheobrominated); C-8 = UIT-1: horti race

= Trinitario and description is crude extracts of UIT-1 (Malaysia) cocoa polyphenols (decaffeinated/detheobrominated));

Fig. 15 L shows the growth inhibition of Hela cells when treated with crude polyphenol extracts obtained from fermented cocoa beans and dried cocoa beans (stages throughout fermentation and sun drying; % control vs. concentration,  $\mu\text{g/ml}$ ; open circle is day zero fraction, darkened circle is day 1 fraction, open inverted triangle is day 2 fraction, darkened inverted triangle is day 3 fraction, open square is day 4 fraction and darkened square is day 9 fraction);

Fig. 15 M shows the effect of enzymically oxidized cocoa procyanidins against Hela cells (dose response for polyphenol oxidase treated crude cocoa polyphenol; % control vs. concentration,  $\mu\text{g/ml}$ ; darkened square is crude UIT-1 (with caffeine and theobromine), open circle crude UIT-1 (without caffeine and theobromine) and darkened circle is crude UIT-1 (polyphenol oxidase catalyzed));

Fig. 15 N shows a representative semi-preparative reverse phase HPLC separation for combined cocoa procyanidin fractions D and E;

Fig. 15 O shows a representative normal phase semi-preparative HPLC separation of a crude cocoa polyphenol extract;

Fig. 16 shows typical Rancimat Oxidation curves for cocoa procyanidin extract and fractions in comparison to the synthetic antioxidants BHA and BHT (arbitrary units vs. time; dotted line and cross (+) is BHA and BHT; \* is D-E; x is crude; open square is A-C; and open diamond is control);

Fig. 17 shows a typical Agarose Gel indicating inhibition of topoisomerase II catalyzed decatenation of kinetoplast DNA by cocoa procyanidin fractions (Lane 1

contains 0.5 $\mu$ g of marker (M) monomer-length kinetoplast DNA circles; Lanes 2 and 20 contain kinetoplast DNA that was incubated with Topoisomerase II in the presence of 4% DMSO, but in the absence of any cocoa procyanidins.

5 (Control -C); Lanes 3 and 4 contain kinetoplast DNA that was incubated with Topoisomerase II in the presence of 0.5 and 5.0  $\mu$ g/mL cocoa procyanidin fraction A; Lanes 5 and 6 contain kinetoplast DNA that was incubated with Topoisomerase II in the presence of 0.5 and 5.0  $\mu$ g/mL  
10 cocoa procyanidin fraction B; Lanes 7, 8, 9, 13, 14 and 15 are replicates of kinetoplast DNA that was incubated with Topoisomerase II in the presence of 0.05, 0.5 and 5.0  $\mu$ g/mL cocoa procyanidin fraction D; Lanes 10, 11, 12, 16, 17 and 18 are replicates of kinetoplast DNA that was  
15 incubated with Topoisomerase II in the presence of 0.05, 0.5, and 5.0  $\mu$ g/mL cocoa procyanidin fraction E; Lane 19 is a replicate of kinetoplast DNA that was incubated with Topoisomerase II in the presence of 5.0  $\mu$ g/mL cocoa procyanidin fraction E);

20 Fig. 18 shows dose response relationships of cocoa procyanidin fraction D against DNA repair competent and deficient cell lines (fractional survival vs.  $\mu$ g/ml; left side xrs-6 DNA Deficient Repair Cell Line, MM-1 D D282X1; right side BR1 Competent DNA Repair Cell Line,  
25 MM-1 D D282B1);

Fig. 19 shows the dose-response curves for Adriamycin resistant MCF-7 cells in comparison to a MCF-7 p168 parental cell line when treated with cocoa fraction D+E (% control vs. concentration,  $\mu$ g/ml; open circle is  
30 MCF-7 p168; darkened circle is MCF-7 ADR); and

Fig. 20 shows the dose-response effects on HeLa cells when treated at 100  $\mu$ g/mL and 25  $\mu$ g/mL levels of twelve fractions prepared by Normal phase semi-preparative HPLC (bar chart, % control vs. control and  
35 fractions 1-12).

DETAILED DESCRIPTION

As discussed above, it has now been surprisingly found that cocoa extracts exhibit anti-cancer, anti-tumor or antineoplastic activity, antioxidant activity and, inhibit DNA topoisomerase II enzyme. The extracts are generally prepared by reducing cocoa beans to a powder, defatting the powder, and extracting the active compound(s) from the defatted powder. The powder can be prepared by freeze-drying the cocoa beans and pulp, depulping the cocoa beans and pulp, dehulling the freeze-dried cocoa beans, and grinding the dehulled beans. The extraction of active compound(s) can be by solvent extraction techniques. The extracts can be purified; for instance, by gel permeation chromatography or by preparative High Performance Liquid Chromatography (HPLC) techniques or by a combination of such techniques. The extracts having activity, without wishing to necessarily be bound by any particular theory, have been identified as cocoa polyphenol(s) such as procyanidins. These cocoa procyanidins have significant anti-cancer, anti-tumor or antineoplastic activity; antioxidant activity; and inhibit DNA topoisomerase II enzyme.

Anti-cancer, anti-tumor or antineoplastic or, antioxidant or DNA topoisomerase II enzyme inhibiting compositions containing the inventive cocoa polyphenols or procyanidins can be prepared in accordance with standard techniques well known to those skilled in the pharmaceutical art. Such compositions can be administered to a patient in need of such administration in dosages and by techniques well known to those skilled in the medical arts taking into consideration such factors as the age, sex, weight, and condition of the particular patient, and the route of administration. The compositions can be co-administered or sequentially administered with other antineoplastic, anti-tumor or

anti-cancer agents or antioxidant or DNA topoisomerase II enzyme inhibiting agents and/or with agents which reduce or alleviate ill effects of antineoplastic, anti-tumor or anti-cancer agents or antioxidant or DNA topoisomerase II  
5 enzyme inhibiting agents; again, taking into consideration such factors as the age, sex, weight, and condition of the particular patient, and, the route of administration.

Examples of compositions of the invention  
10 include solid compositions for oral administration such as capsules, tablets, pills and the like, as well as chewable solid formulations, to which the present invention may be well-suited since it is from an edible source (e.g., cocoa or chocolate flavored solid  
15 compositions); liquid preparations for orifice, e.g., oral, nasal, anal, vaginal etc., administration such as suspensions, syrups or elixirs; and, preparations for parental, subcutaneous, intradermal, intramuscular or intravenous administration (e.g., injectable  
20 administration) such as sterile suspensions or emulsions. However, the active ingredient in the compositions may complex with proteins such that when administered into the bloodstream, clotting may occur due to precipitation of blood proteins; and, the skilled artisan should take  
25 this into account. In such compositions the active cocoa extract may be in admixture with a suitable carrier, diluent, or excipient such as sterile water, physiological saline, glucose or the like. The active cocoa extract of the invention can be provided in  
30 lyophilized form for reconstituting, for instance, in isotonic aqueous, saline buffer.

Further, the invention also comprehends a kit wherein the active cocoa extract is provided. The kit can include a separate container containing a suitable  
35 carrier, diluent or excipient. The kit can also include

an additional anti-cancer, anti-tumor or antineoplastic agent or antioxidant or DNA topoisomerase II enzyme inhibiting agent and/or an agent which reduces or alleviates ill effects of antineoplastic, anti-tumor or  
5 anti-cancer agents or antioxidant or DNA topoisomerase II enzyme inhibiting agents for co- or sequential-administration. The additional agent(s) can be provided in separate container(s) or in admixture with the active cocoa extract. Additionally, the kit can include  
10 instructions for mixing or combining ingredients and/or administration.

Furthermore, while the invention is described with respect to cocoa extracts preferably comprising cocoa procyanidins, from this disclosure the skilled  
15 organic chemist will appreciate and envision synthetic routes to obtain the active compounds. Accordingly, the invention comprehends synthetic cocoa polyphenols or procyanidins or their derivatives which include, but are not limited to glycosides, gallates, esters, etc. and the  
20 like.

The following non-limiting Examples are given by way of illustration only and are not to be considered a limitation of this invention, many apparent variations of which are possible without departing from the spirit  
25 or scope thereof.

EXAMPLES**Example 1: Cocoa Source and Method of Preparation**

Several *Theobroma cacao* genotypes which represent the three recognized horticultural races of cocoa (Enriquez, 1967; Engels, 1981) were obtained from the three major cocoa producing origins of the world. A list of those genotypes used in this study are shown in Table 1. Harvested cocoa pods were opened and the beans with pulp were removed for freeze drying. The pulp was manually removed from the freeze dried mass and the beans were subjected to analysis as follows. The unfermented, freeze dried cocoa beans were first manually dehulled, and ground to a fine powdery mass with a TEKMAR Mill. The resultant mass was then defatted overnight by Soxhlet extraction using redistilled hexane as the solvent. Residual solvent was removed from the defatted mass by vacuum at ambient temperature.



Table 1: Description of *Theobroma cacao* Source Material

GENOTYPE	ORIGIN	HORTICULTURAL RACE
UIT-1	Malaysia	Trinitario
Unknown	West Africa	Forastero
ICS-100	Brazil	Trinitario
ICS-39	Brazil	Trinitario
UF-613	Brazil	Trinitario
EEG-48	Brazil	Forastero
UF-12	Brazil	Criollo
NA-33	Brazil	Forastero

Example 2: Procyanidin Extraction ProceduresA. Method 1

Procyanidins were extracted from the defatted, unfermented, freeze dried cocoa beans of Example 1 using a modification of the method described by Jalal and Collin (1977). Procyanidins were extracted from 50 gram batches of the defatted cocoa mass with 2X 400 mL 70% acetone/deionized water followed by 400mL 70% methanol/deionized water. The extracts were pooled and the solvents removed by evaporation at 45°C with a rotary evaporator held under partial vacuum. The resultant aqueous phase was diluted to 1L with deionized water and extracted 2X with 400mL CHCl<sub>3</sub>. The solvent phase was discarded. The aqueous phase was then extracted 4X with 500mL ethyl acetate. Any resultant emulsions were broken by centrifugation on a Sorvall RC 28S centrifuge operated at 2,000 xg for 30 min. at 10°C. To the combined ethyl acetate extracts, 100-200mL deionized water was added. The solvent was removed by evaporation at 45°C with a rotary evaporator held under partial vacuum. The resultant aqueous phase was frozen in liquid N<sub>2</sub> followed by freeze drying on a LABCONCO Freeze Dry System. The yields of crude procyanidins that were obtained from the different cocoa genotypes are listed in Table 2.

Table 2: Crude Procyanidin Yields

GENOTYPE	ORIGIN	YIELDS (g)
UIT-1	Malaysia	3.81
Unknown	West Africa	2.55
ICS-100	Brazil	3.42
ICS-39	Brazil	3.45
UF-613	Brazil	2.98
EEG-48	Brazil	3.15
UF-12	Brazil	1.21
NA-33	Brazil	2.23

B. Method 2

Alternatively, procyanidins are extracted from defatted, unfermented, freeze dried cocoa beans of Example 1 with 70% aqueous acetone. Ten grams of defatted material was slurried with 100 mL solvent for 5-10 min. The slurry was centrifuged for 15 min. at 4°C at 3000 xg and the supernatant passed through glass wool. The filtrate was subjected to distillation under partial vacuum and the resultant aqueous phase frozen in liquid N<sub>2</sub>, followed by freeze drying on a LABCONCO Freeze Dry System. The yields of crude procyanidins ranged from 15-20%.

Without wishing to be bound by any particular theory, it is believed that the differences in crude yields reflected variations encountered with different genotypes, geographical origin, horticultural race, and method of preparation.

Example 3: Partial Purification of Cocoa ProcyanidinsA. Gel Permeation Chromatography

Procyanidins obtained from Example 2 were partially purified by liquid chromatography on Sephadex LH-20 (28 x 2.5 cm). Separations were aided by a step gradient into deionized water. The initial gradient

composition started with 15% methanol in deionized water which was followed step wise every 30 min. with 25% methanol in deionized water, 35% methanol in deionized water, 70% methanol in deionized water, and finally 100% methanol. The effluent following the elution of the xanthine alkaloids (caffeine and theobromine) was collected as a single fraction. The fraction yielded a xanthine alkaloid free subfraction which was submitted to further subfractionation to yield five subfractions designated MM2A through MM2E. The solvent was removed from each subfraction by evaporation at 45°C with a rotary evaporator held under partial vacuum. The resultant aqueous phase was frozen in liquid N<sub>2</sub> and freeze dried overnight on a LABCONCO Freeze Dry System. A representative gel permeation chromatogram showing the fractionation is shown in Figure 1. Approximately, 100mg of material was subfractionated in this manner.

Figure 1: Gel Permeation Chromatogram of Crude Procyanidins on Sephadex LH-20

Chromatographic Conditions: Column; 28 x 2.5 cm Sephadex LH-20, Mobile Phase: Methanol/Water Step Gradient, 15:85, 25:75, 35:65, 70:30, 100:0 Stepped at 1/2 Hour Intervals, Flow Rate; 1.5ml/min, Detector; UV @  $\lambda_1=254$  nm and  $\lambda_2=365$  nm, Chart Speed: 0.5 mm/min, Column Load; 120 mg.

B. Semi-preparative High Performance Liquid Chromatography (HPLC)

Method 1: Reverse Phase Separation

Procyanidins obtained from Example 2 and/or 3A were partially purified by semi-preparative HPLC. A Hewlett Packard 1050 HPLC System equipped with a variable wavelength detector, Rheodyne 7010 injection valve with 1 mL injection loop was assembled with a Pharmacia FRAC-100 Fraction Collector. Separations were effected on a

Phenomenex Ultracarb 10  $\mu$  ODS column (250 x 22.5 mm) connected with a Phenomenex 10  $\mu$  ODS Ultracarb (60 x 10 mm) guard column. The mobile phase composition was A = water; B = methanol used under the following linear gradient conditions: [Time, %A]; (0,85), (60,50), (90,0), and (110,0) at a flow rate of 5 mL/min.

A representative Semi-preparative HPLC trace is shown in Figure 15N for the separation of procyanidins present in fraction D + E. Individual peaks or select chromatographic regions were collected on timed intervals or manually by fraction collection for further purification and subsequent evaluation. Injection loads ranged from 25-100 mg of material.

Method 2. Normal Phase Separation

Procyanidin extracts obtained from Examples 2 and/or 3A were partially purified by semi-preparative HPLC. A Hewlett Packard 1050 HPLC system, Millipore-Waters Model 480 LC detector set at 254 nm was assembled with a Pharmacia Frac-100 Fraction Collector set in peak mode. Separations were effected on a Supelco 5 $\mu$  Supelcosil LC-Si column (250 x 10mm) connected with a Supelco 5 $\mu$  Supelguard LC-Si guard column (20 x 4.6mm). Procyanidins were eluted by a linear gradient under the following conditions: (Time, %A, %B); (0,82,14), (30, 67.6, 28.4), (60, 46, 50), (65, 10, 86), (70, 10, 86) followed by a 10 min. re-equilibration. Mobile phase composition was A=dichloromethane; B=methanol; and C=acetic acid: water (1:1). A flow rate of 3 mL/min was used. Components were detected by UV at 254 nm, and recorded on a Kipp & Zonan BD41 recorder. Injection volumes ranged from 100-250  $\mu$ L of 10mg of procyanidin extracts dissolved in 0.25 mL 70% aqueous acetone. A representative semi-preparative HPLC trace is shown in Figure 15 O. Individual peaks or select chromatographic regions were collected on timed intervals or manually by

fraction collection for further purification and subsequent evaluation.

5 HPLC Conditions: 250 x 10mm Supelco Supelcosil LC-Si  
(5 $\mu$ m) Semipreparative Column  
20 x 4.6mm Supelco Supelcosil LC-Si  
(5 $\mu$ m) Guard Column

10 Detector: Waters LC  
Spectrophotometer Model  
480 @ 254nm

15 Flow rate: 3mL/min,  
Column Temperature: ambient,  
Injection: 250 $\mu$ L of 70% aqueous  
acetone extract.

Gradient: Time (min)	CH <sub>2</sub> Cl <sub>2</sub>	Methanol	Acetic Acid/H <sub>2</sub> O (1:1)
0	82	14	4
20 30	67.6	28.4	4
60	46	50	4
65	10	86	4
70	10	86	4

25 The fractions obtained were as follows:

FRACTION	TYPE
1	dimers
2	trimers
30 3	tetramers
4	pentamers
5	hexamers
6	heptamers
7	octamers
35 8	nonamers
9	decamers
10	undecamers
11	dodecamers
12	higher oligomers

Example 4: Analytical HPLC Analysis of Procyanidin  
Extracts

Method 1: Reverse Phase Separation

5 Procyanidin extracts obtained from Example 3  
were filtered through a  $0.45\mu$  filter and analyzed by a  
Hewlett Packard 1090 ternary HPLC system equipped with a  
Diode Array detector and a HP model 1046A Programmable  
Fluorescence Detector. Separations were effected at  $45^{\circ}\text{C}$   
10 on a Hewlett-Packard  $5\mu$  Hypersil ODS column ( $200 \times$   
 $2.1\text{mm}$ ). The flavanols and procyanidins were eluted with  
a linear gradient of 60% B into A followed by a column  
wash with B at a flow rate of  $0.3\text{mL/min}$ . The mobile  
phase composition was B= 0.5% acetic acid in methanol and  
15 A= 0.5% acetic acid in deionized water. Acetic acid  
levels in A and B mobile phases can be increased to 2%.  
Components were detected by fluorescence, where  $\lambda_{\text{ex}} =$   
 $276\text{nm}$  and  $\lambda_{\text{em}} = 316\text{nm}$ . Concentrations of (+)-catechin  
and (-)-epicatechin were determined relative to reference  
20 standard solutions. Procyanidin levels were estimated by  
using the response factor for (-)-epicatechin. A  
representative HPLC chromatogram showing the separation  
of the various components is shown in Figure 2A for one  
cocoa genotype. Similar HPLC profiles were obtained from  
25 the other cocoa genotypes.

HPLC Conditions: Column: 200 x 2.1mm Hewlett Packard  
Hypersil ODS (5 $\mu$ )  
Guard column: 20 x 2.1mm Hewlett  
Packard Hypersil ODS (5 $\mu$ )  
Detectors: Diode Array @ 289nm  
Fluorescence  $\lambda_{ex}$  = 276nm;  
 $\lambda_{em}$  = 316nm.  
Flow rate: 3mL/min.  
Column Temperature: 45°C

Gradient: Time (min)	0.5% Acetic Acid in deionized water	0.5% Acetic acid in methanol
0	100	0
50	40	60
60	0	100

#### Method 2: Normal Phase Separation

Procyanidin extracts obtained from Examples 2 and/or 3 were filtered through a 0.45  $\mu$  filter and analyzed by a Hewlett Packard 1090 Series II HPLC system equipped with a HP model 1046A Programmable Fluorescence detector and Diode Array detector. Separations were effected at 37°C on a 5 $\mu$  Phenomenex Lichrosphere Silica 100 column (250 x 3.2mm) connected to a Supelco Supelguard LC-Si 5 $\mu$  guard column (20 x 4.6mm). Procyanidins were eluted by linear gradient under the following conditions: (Time, %A, %B); (0, 82, 14), (30, 67.6, 28.4), (60, 46, 50), (65, 10, 86), (70, 10, 86) followed by an 8 min. re-equilibration. Mobile phase composition was A=dichloromethane, B=methanol, and C=acetic acid: water at a volume ratio of 1:1. A flow rate of 0.5 mL/min. was used. Components were detected by fluorescence, where  $\lambda_{ex}$  = 276nm and  $\lambda_{em}$  = 316nm or by UV at 280 nm. A representative HPLC chromatogram showing the separation of the various procyanidins is shown in Figure 2B for one genotype. Similar HPLC profiles were obtained from other cocoa genotypes.

**HPLC Conditions:**

5 250 x 3.2mm Phenomenex Lichrosphere Silica 100  
column (5 $\mu$ ) 20 x 4.6mm Supelco Supelguard LC-Si  
(5 $\mu$ ) guard column  
Detectors: Photodiode Array @ 280nm  
Fluorescence  $\lambda_{ex}$  = 276nm;  
10  $\lambda_{em}$  = 316nm.  
Flow rate: 0.5 mL/min.  
Column Temperature: 37°C

Gradient: Time (min.)	CH <sub>2</sub> -Cl <sub>2</sub>	Methanol	Acetic Acid/Water (1:1)
15 0	82	14	4
30	67.6	28.4	4
60	46	50	4
65	10	86	4
20 70	10	86	4

**Example 5: Identification of Procyanidins**

Procyanidins were purified by liquid  
chromatography on Sephadex LH-20 (28 x 2.5cm) columns  
followed by semi-preparative HPLC using a 10 $\mu$   $\mu$ Bondapak  
25 C18 (100 x 8mm) column or by semi-preparative HPLC using  
a 5 $\mu$  Supelcosil LC-Si (250 x 10mm) column.

Partially purified isolates were analyzed by  
Fast Atom Bombardment - Mass Spectrometry (FAB-MS) on a  
VG ZAB-T high resolution MS system using a Liquid  
30 Secondary Ion Mass Spectrometry (LSIMS) technique in  
positive and negative ion modes. A cesium ion gun was  
used as the ionizing source at 30kV and a "Magic Bullet  
Matrix" (1:1 dithiothreitol/dithioerythritol) was used as  
the proton donor.

35 Analytical investigations of these fractions by  
LSIMS revealed the presence of a number of flavan-3-ol  
oligomers as shown in Table 3.



Table 3: LSIMS (Positive Ion) Data from Cocoa  
Procyanidin Fractions

Oligomer	(M + 1) <sup>+</sup> m/z	(M + Na) <sup>+</sup> m/z	Mol. Wt.
Monomers (catechins)	291	313	290
Dimer(s)	577/579	599/601	576/578
Trimer(s)	865/867	887/889	884/866
Tetramer(s)	1155	1177	1154
Pentamer(s)	1443	1465	1442
Hexamer(s)	1731	1753	1730
Heptamer(s)	---	2041	2018
Octamer(s)	---	2329	2306
Nonamer(s)	---	2617	2594
Decamer(s)	---	2905	2882
Undecamer(s)	---	---	3170
Dodecamer(s)	---	---	3458

The major mass fragment ions were consistent with work previously reported for both positive and negative ion FAB-MS analysis of procyanidins (Self et al., 1986 and Porter et al., 1991). The ion corresponding to  $m/z$  577  $(M+H)^+$  and its sodium adduct at  $m/z$  599  $(M+Na)^+$  suggested the presence of doubly linked procyanidin dimers in the isolates. It was interesting to note that the higher oligomers were more likely to form sodium adducts  $(M+Na)^+$  than their protonated molecular ions  $(M+H)^+$ . The procyanidin isomers B-2, B-5 and C-1 were tentatively identified based on the work reported by Revilla et al. (1991), Self et al. (1986) and Porter et al. (1991). Procyanidins up to both the octamer and decamer were verified by FAB-MS in the partially purified fractions. Additionally, evidence for procyanidins up to the dodecamer were observed from

normal phase HPLC analysis (see Figure 2B). Without wishing to be bound by any particular theory, it is believed that the dodecamer is the limit of solubility in the solvents used in the extraction and purification

5 schemes. Table 4 lists the relative concentrations of the procyanidins found in xanthine alkaloid free isolates based on reverse phase HPLC analysis. Table 5 lists the relative concentrations of the procyanidins based on normal phase HPLC analysis.

10 Table 4: Relative Concentrations of Procyanidins in the Xanthine Alkaloid Free Isolates

	Component	Molecular Weight	Amount
	(+)-catechin	290	1.6%
15	(-)-epicatechin	290	38.2%
	B-2 Dimer	578	11.0%
	B-5 Dimer	578	5.3%
	C-1 Trimer	866	9.3%
	Doubly linked dimers	576	3.0%
20	Tetramer(s)	1154	4.5%
	Pentamer-Octamer	1442-2306	24.5%
	Unknowns and higher oligomers	---	2.6%

Table 5: Relative Concentrations of Procyanidins in  
Aqueous Acetone Extracts

5	Component	Molecular Weight	Amount
	(+)-catechin and (-) -epicatechin	290 (same for each)	41.9%
	B-2 and B-5 Dimers	578	13.9%
	Trimers	884/866	11.3%
10	Tetramers	1154	9.9%
	Pentamers	1442	7.8%
	Hexamers	1730	5.1%
	Heptamers	2018	4.2%
	Octamers	2306	2.8%
15	Nonamers	2594	1.6%
	Decamers	2882	0.7%
	Undecamers	3170	0.2%
	Dodecamers	3458	<0.1%

20 Figure 3 shows several procyanidin structures  
and Figures 4A-4E show the representative HPLC  
chromatograms of the five fractions employed in the  
following screening for anti-cancer or antineoplastic  
activity. The HPLC conditions for Figs. 4A-4E were as  
25 follows:

HPLC Conditions: Hewlett Packard 1090 ternary  
HPLC System equipped with HP Model 1046A  
Programmable Fluorescence Detector.

30 Column: Hewlett Packard 5 $\mu$  Hypersil ODS (200 x  
2.1 mm) Linear Gradient of 60% B into A at a  
flow rate of 0.3 ml/min. B = 0.5% acetic acid  
in methanol; A = 0.5% acetic acid in deionized  
water.  $\lambda_{ex}$  = 280nm;  $\lambda_{em}$  = 316nm.

Figure 15 O shows a representative semi-prep  
35 HPLC chromatogram of an additional 12 fractions employed

in the screening for anticancer or antineoplastic activity (HPLC conditions stated above).

Example 6: Anti-Cancer, Anti-Tumor or Antineoplastic Activity of Cocoa Extracts (Procyanidins)

5

The MTT (3-[4,5-dimethyl thiazol-2yl]-2,5-diphenyltetrazolium bromide) - microtiter plate tetrazolium cytotoxicity assay originally developed by Mosmann (1983) was used to screen test samples from

10 Example 5. Test samples, standards (cisplatin and chlorambucil) and MTT reagent were dissolved in 100% DMSO (dimethyl sulfoxide) at a 10 mg/mL concentration. Serial dilutions were prepared from the stock solutions. In the case of the test samples, dilutions ranging from 0.01  
15 through 100  $\mu$ g/mL were prepared in 0.5% DMSO.

All human tumor cell lines were obtained from the American Type Culture Collection. Cells were grown as mono layers in alpha-MEM containing 10% fetal bovine serum, 100 units/mL penicillin, 100  $\mu$ g/mL streptomycin  
20 and 240 units/mL nystatin. The cells were maintained in a humidified, 5% CO<sub>2</sub> atmosphere at 37°C.

After trypsinization, the cells are counted and adjusted to a concentration of  $50 \times 10^5$  cells/mL (varied according to cancer cell line). 200 $\mu$ L of the cell  
25 suspension was plated into wells of 4 rows of a 96-well microtiter plate. After the cells were allowed to attach for four hours, 2 $\mu$ L of DMSO containing test sample solutions were added to quadruplicate wells. Initial dose-response finding experiments, using order of  
30 magnitude test sample dilutions were used to determine the range of doses to be examined. Well absorbencies at 540nm were then measured on a BIO RAD MP450 plate reader. The mean absorbance of quadruplicate test sample treated wells was compared to the control, and the results.  
35 expressed as the percentage of control absorbance plus/minus the standard deviation. The reduction of MTT

to a purple formazan product correlates in a linear manner with the number of living cells in the well. Thus, by measuring the absorbance of the reduction product, a quantitation of the percent of cell survival  
5 at a given dose of test sample can be obtained. Control wells contained a final concentration of 1% DMSO.

Two of the samples were first tested by this protocol. Sample MM1 represented a very crude isolate of cocoa procyanidins and contained appreciable quantities  
10 of caffeine and theobromine. Sample MM2 represented a cocoa procyanidin isolate partially purified by gel permeation chromatography. Caffeine and theobromine were absent in MM2. Both samples were screened for activity against the following cancer cell lines using the  
15 procedures previously described:

HCT 116 colon cancer  
ACHN renal adenocarcinoma  
SK-5 melanoma  
A498 renal adenocarcinoma  
20 MCF-7 breast cancer  
PC-3 prostate cancer  
CAPAN-2 pancreatic cancer

Little or no activity was observed with MM1 on any of the cancer cell lines investigated. MM2 was found  
25 to have activity against HCT-116, PC-3 and ACHN cancer cell lines. However, both MM1 and MM2 were found to interfere with MTT such that it obscured the decrease in absorbance that would have reflected a decrease in viable cell number. This interference also contributed to large  
30 error bars, because the chemical reaction appeared to go more quickly in the wells along the perimeter of the plate. A typical example of these effects is shown in Figure 5. At the high concentrations of test material, one would have expected to observe a large decrease in  
35 survivors rather than the high survivor levels shown. Nevertheless, microscopic examinations revealed that

cytotoxic effects occurred, despite the MTT interference effects. For instance, an  $IC_{50}$  value of 0.5  $\mu\text{g/mL}$  for the effect of MM2 on the ACHN cell line was obtained in this manner.

5           These preliminary results, in the inventors' view, required amendment of the assay procedures to preclude the interference with MTT. This was accomplished as follows. After incubation of the plates at 37°C in a humidified, 5%  $\text{CO}_2$  atmosphere for 18 hours,  
10 the medium was carefully aspirated and replaced with fresh alpha-MEM media. This media was again aspirated from the wells on the third day of the assay and replaced with 100 $\mu\text{L}$  of freshly prepared McCoy's medium. 11 $\mu\text{L}$  of a 5mg/mL stock solution of MTT in PBS (Phosphate Buffered  
15 Saline) were then added to the wells of each plate. After incubation for 4 hours in a humidified, 5%  $\text{CO}_2$  atmosphere at 37°C, 100 $\mu\text{L}$  of 0.04 N HCl in isopropanol was added to all wells of the plate, followed by thorough mixing to solubilize the formazan produced by any viable  
20 cells. Additionally, it was decided to subfractionate the procyanidins to determine the specific components responsible for activity.

          The subfractionation procedures previously described were used to prepare samples for further  
25 screening. Five fractions representing the areas shown in Figure 1 and component(s) distribution shown in Figures 4A - 4E were prepared. The samples were coded MM2A through MM2E to reflect these analytical characterizations and to designate the absence of  
30 caffeine and theobromine.

          Each fraction was individually screened against the HCT-116, PC-3 and ACHN cancer cell lines. The results indicated that the activity did not concentrate to any one specific fraction. This type of result was  
35 not considered unusual, since the components in "active" natural product isolates can behave synergistically. In

the case of the cocoa procyanidin isolate (MM2), over twenty detectable components comprised the isolate. It was considered possible that the activity was related to a combination of components present in the different  
5 fractions, rather than the activity being related to an individual component(s).

On the basis of these results, it was decided to combine the fractions and repeat the assays against the same cancer cell lines. Several fraction  
10 combinations produced cytotoxic effects against the PC-3 cancer cell lines. Specifically,  $IC_{50}$  values of 40  $\mu\text{g/mL}$  each for MM2A and MM2E combination, and of 20  $\mu\text{g/mL}$  each for MM2C and MM2E combination, were obtained. Activity was also reported against the HCT-116 and ACHN cell  
15 lines, but as before, interference with the MTT indicator precluded precise observations. Replicate experiments were repeatedly performed on the HCT-116 and ACHN lines to improve the data. However, these results were inconclusive due to bacterial contamination and  
20 exhaustion of the test sample material. Figures 6A-6D show the dose-response relationship between combinations of the cocoa extracts and PC-3 cancer cells.

Nonetheless, from this data, it is clear that cocoa extracts, especially cocoa polyphenols or  
25 procyanidins, have significant anti-tumor; anti-cancer or antineoplastic activity, especially with respect to human PC-3 (prostate), HCT-116 (colon) and ACHN (renal) cancer cell lines. In addition, those results suggest that specific procyanidin fractions may be responsible for the  
30 activity against the PC-3 cell line.

Example 7: Anti-Cancer, Anti-Tumor or Antineoplastic Activity of Cocoa Extracts (Procyanidins)

To confirm the above findings and further study  
35 fraction combinations, another comprehensive screening was performed.

All prepared materials and procedures were identical to those reported above, except that the standard 4-replicates per test dose was increased to 8 or 12-replicates per test dose. For this study, individual and combinations of five cocoa procyanidin fractions were screened against the following cancer cell lines.

PC-3 Prostate  
 KB Nasopharyngeal/HeLa  
 HCT-116 Colon  
 10 ACHN Renal  
 MCF-7 Breast  
 SK-5 Melanoma  
 A-549 Lung  
 CCRF-CEM T-cell leukemia

15 Individual screenings consisted of assaying different dose levels (0.01-100 $\mu$ g/mL) of fractions A, B, C, D, and E (See Figs. 4A-4E and discussion thereof, supra) against each cell line. Combination screenings consisted of combining equal dose levels of fractions  
 20 A+B, A+C, A+D, A+E, B+C, B+D, B+E, C+D, C+E, and D+E against each cell line. The results from these assays are individually discussed, followed by an overall summary.

A. PC-3 Prostate Cell Line

25 Figures 7A - 7H show the typical dose response relationship between cocoa procyanidin fractions and the PC-3 cell line. Figures 7D and 7E demonstrate that fractions D and E were active at an IC<sub>50</sub> value of 75  $\mu$ g/mL. The IC<sub>50</sub> values that were obtained from dose-  
 30 response curves of the other procyanidin fraction combinations ranged between 60 - 80  $\mu$ g/mL when fractions D or E were present. The individual IC<sub>50</sub> values are listed in Table 6.

B. KB Nasopharyngeal/HeLa Cell Line

35 Figures 8A - 8H show the typical dose response relationship between cocoa procyanidin fractions and the



KB Nasopharyngeal/HeLa cell line. Figures 8D and 8E demonstrate that fractions D and E were active at an  $IC_{50}$  value of 75  $\mu\text{g/mL}$ . Figures 8F - 8H depict representative results obtained from the fraction combination study. In this case, procyanidin fraction combination A+B had no effect, whereas fraction combinations B+E and D+E were active at an  $IC_{50}$  value of 60  $\mu\text{g/mL}$ . The  $IC_{50}$  values that were obtained from other dose response curves from other fraction combinations ranged from 60 - 80  $\mu\text{g/mL}$  when fractions D or E were present. The individual  $IC_{50}$  values are listed in Table 6. These results were essentially the same as those obtained against the PC-3 cell line.

#### C. HCT-116 Colon Cell Line

Figure 9A - 9H show the typical dose response relationships between cocoa procyanidin fractions and the HCT-116 colon cell line. Figures 9D and 9E demonstrate that fraction E was active at an  $IC_{50}$  value of approximately 400  $\mu\text{g/mL}$ . This value was obtained by extrapolation of the existing curve. Note that the slope of the dose response curve for fraction D also indicated activity. However, no  $IC_{50}$  value was determined from this plot, since the slope of the curve was too shallow to obtain a reliable value. Figures 9F - 9H depict representative results obtained from the fraction combination study. In this case, procyanidin fraction combination B+D did not show appreciable activity, whereas fraction combinations A+E and D+E were active at  $IC_{50}$  values of 500  $\mu\text{g/mL}$  and 85  $\mu\text{g/mL}$ , respectively. The  $IC_{50}$  values that were obtained from dose response curves of other fraction combinations averaged about 250  $\mu\text{g/mL}$  when fraction E was present. The extrapolated  $IC_{50}$  values are listed in Table 6.

#### D. ACHN Renal Cell Line

Figure 10A - 10H show the typical dose response relationships between cocoa procyanidin fractions and the ACHN renal cell line. Figures 10A - 10E indicated that

no individual fraction was active against this cell line. Figures 10F - 10H depict representative results obtained from the fraction combination study. In this case, procyanidin fraction combination B+C was inactive, whereas the fraction combination A+E resulted in an extrapolated  $IC_{50}$  value of approximately 500  $\mu\text{g/mL}$ . Dose response curves similar to the C+D combination were considered inactive, since their slopes were too shallow. Extrapolated  $IC_{50}$  values for other fraction combinations are listed in Table 6.

#### E. A-549 Lung Cell Line

Figures 11A - 11H show the typical dose response relationships between cocoa procyanidin fractions and the A-549 lung cell line. No activity could be detected from any individual fraction or combination of fractions at the doses used in the assay. However, procyanidin fractions may nonetheless have utility with respect to this cell line.

#### F. SK-5 Melanoma Cell Line

Figure 12A - 12H show the typical dose response relationships between cocoa procyanidin fractions and the SK-5 melanoma cell line. No activity could be detected from any individual fraction or combination of fractions at the doses used in the assay. However, procyanidin fractions may nonetheless have utility with respect to this cell line.

#### G. MCF-7 Breast Cell Line

Figures 13A - 13H show the typical dose response relationships between cocoa procyanidin fractions and the MCF-7 breast cell line. No activity could be detected from any individual fraction or combination of fractions at the doses used in the assay. However, procyanidin fractions may nonetheless have utility with respect to this cell line.

#### H. CCRF-CEM T-Cell Leukemia Line

Atypical dose response curves were originally obtained against the CCRF-CEM T-cell leukemia line.

However, microscopic counts of cell number versus time at different fraction concentrations indicated that 500  $\mu$ g of fractions A, B and D effected an 80% growth reduction over a four day period. A representative dose response relationship is shown in Figure 14.

#### I. Summary

10 The  $IC_{50}$  values obtained from these assays are collectively listed in Table 6 for all the cell lines except for CCRF-CEM T-cell leukemia. The T-cell leukemia data was intentionally omitted from the Table, since a different assay procedure was used. A general summary of  
15 these results indicated that the most activity was associated with fractions D and E. These fractions were most active against the PC-3 (prostate) and KB (nasopharyngeal/HeLa) cell lines. These fractions also evidenced activity against the HCT-116 (colon) and ACHN  
20 (renal) cell lines, albeit but only at much higher doses. No activity was detected against the MCF-7 (breast), SK-5 (melanoma) and A-549 (lung) cell lines. However, procyanidin fractions may nonetheless have utility with respect to these cell lines. Activity was also shown  
25 against the CCRF-CEM (T-cell leukemia) cell line. It should also be noted that fractions D and E are the most complex compositionally. Nonetheless, from this data it is clear that cocoa extracts, especially cocoa procyanidins, have significant anti-tumor, anti-cancer or  
30 antineoplastic activity.

Table 6: IC<sub>50</sub> Values for Cocoa Procyanidin Fractions  
Against Various Cell Lines

(IC<sub>50</sub> values in µg/mL)

5

10

15

20

FRACTION	PC-3	KB	HCT-116	ACHN	MCF-7	SK-5	A-549
A							
B							
C							
D	90	80					
E	75	75	400				
A+B							
A+C	125	100					
A+D	75	75					
A+E	80	75	500	500			
B+C							
B+D	75	80					
B+E	60	65	200				
C+D	80	75		1000			
C+E	80	70	250				
D+E	80	60	85				

Values above 100 µg/mL were extrapolated from  
dose response curves

25 **Example 8. Anti-Cancer, Anti-Tumor or Antineoplastic  
Activity of Cocoa Extracts (Procyanidins)**

Several additional in vitro assay procedures  
were used to complement and extend the results presented  
30 in Examples 6 and 7.

**Method A. Crystal Violet Staining Assay**

All human tumor cell lines were obtained from  
the American Type Culture Collection. Cells were grown  
35 as monolayers in IMEM containing 10% fetal bovine serum  
without antibiotics. The cells were maintained in a  
humidified, 5% CO<sub>2</sub> atmosphere at 37°C.

After trypsinization, the cells were counted  
and adjusted to a concentration of 1,000-2,000 cells per

100  $\mu$ L. Cell proliferation was determined by plating the cells (1,000-2,000 cells/well) in a 96 well microtiter plate. After addition of 100  $\mu$ L cells per well, the cells were allowed to attach for 24 hours. At the end of the 24 hour period, various cocoa fractions were added at different concentrations to obtain dose response results. The cocoa fractions were dissolved in media at a 2 fold concentration and 100  $\mu$ L of each solution was added in triplicate wells. On consecutive days, the plates were stained with 50  $\mu$ L crystal violet (2.5g crystal violet dissolved in 125 mL methanol, 375 mL water), for 15 min. The stain was removed and the plate was gently immersed into cold water to remove excess stain. The washings were repeated two more times, and the plates allowed to dry. The remaining stain was solubilized by adding 100  $\mu$ L of 0.1 M sodium citrate/50% ethanol to each well. After solubilization, the number of cells were quantitated on an ELISA plate reader at 540nm (reference filter at 410nm). The results from the ELISA reader were graphed with absorbance on the y-axis and days growth on the x-axis.

#### Method B. Soft Agar Cloning Assay

Cells were cloned in soft agar according to the method described by Nawata et al. (1981). Single cell suspensions were made in media containing 0.8% agar with various concentrations of cocoa fractions. The suspensions were aliquoted into 35mm dishes coated with media containing 1.0% agar. After 10 days incubation, the number of colonies greater than 60  $\mu$ m in diameter were determined on an Ominicron 3600 Image Analysis System. The results were plotted with number of colonies on the y-axis and the concentrations of a cocoa fraction on the x-axis.

#### Method C. XTT-Microculture Tetrazolium Assay

The XTT assay procedure described by Scudiero et al. (1988) was used to screen various cocoa fractions. The XTT assay was essentially the same as that described using the MTT procedure (Example 6) except for the

following modifications. XTT ((2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) was prepared at 1mg/mL medium without serum, prewarmed to 37°C. PMS was prepared at 5mM PBS. XTT and PMS were mixed together; 10μL of PMS per mL XTT and 50μL PMS-XTT were added to each well. After an incubation at 37°C for 4 hr, the plates were mixed 30 min. on a mechanical shaker and the absorbance measured at 450-600nm. The results were plotted with the absorbance on the y-axis and days growth or concentration on the x-axis.

For methods A and C, the results were also plotted as the percent control as the y-axis and days growth or concentration on the x-axis.

A comparison of the XTT and Crystal Violet Assay procedures was made with cocoa fraction D & E (Example 3B) against the breast cancer cell line MCF-7 p168 to determine which assay was most sensitive. As shown in Figure 15A, both assays showed the same dose-response effects for concentrations >75 μg/mL. At concentrations below this value, the crystal violet assay showed higher standard deviations than the XTT assay results. However, since the crystal violet assay was easier to use, all subsequent assays, unless otherwise specified, were performed by this procedure.

Crystal violet assay results are presented (Figures 15B-15E) to demonstrate the effect of a crude polyphenol extract (Example 2) on the breast cancer cell line MDA MB231, prostate cancer cell line PC-3, breast cancer cell line MCF-7 p163, and cervical cancer cell line Hela, respectively. In all cases a dose of 250μg/mL completely inhibited all cancer cell growth over a period of 5-7 days. The Hela cell line appeared to be more sensitive to the extract, since a 100μg/mL dose also inhibited growth. Cocoa fractions from Example 3B were also assayed against Hela and another breast cancer cell line SKBR-3. The results (Figures 15F and 15G) showed that fraction D & E has the highest activity. As shown

in Figures 15H and 15I,  $IC_{50}$  values of about  $40\mu\text{g/mL}$  D & E were obtained from both cancer cell lines.

The cocoa fraction D & E was also tested in the soft agar cloning assay which determines the ability of a test compound(s) to inhibit anchorage independent growth. As shown in Figure 15J, a concentration of  $100\mu\text{g/mL}$  completely inhibited colony formation of Hela cells.

Crude polyphenol extracts obtained from eight different cocoa genotypes representing the three horticultural races of cocoa were also assayed against the Hela cell line. As shown in Figure 15K all cocoa varieties showed similar dose-response effects. The UIT-1 variety exhibited the most activity against the Hela cell line. These results demonstrated that all cocoa genotypes possess a polyphenol fraction that elicits activity against at least one human cancer cell line that is independent of geographical origin, horticultural race, and genotype.

Another series of assays were performed on crude polyphenol extracts prepared on a daily basis from a one ton scale traditional 5-day fermentation of Brazilian cocoa beans, followed by a 4-day sun drying stage. The results shown in Figure 15L showed no obvious effect of these early processing stages, suggesting little change in the composition of the polyphenols. However, it is known (Lehrian and Patterson, 1983) that polyphenol oxidase (PPO) will oxidize polyphenols during the fermentation stage. To determine what effect enzymically oxidized polyphenols would have on activity, another experiment was performed. Crude PPO was prepared by extracting finely ground, unfermented, freeze dried, defatted Brazilian cocoa beans with acetone at a ratio of 1 gm powder to 10 mL acetone. The slurry was centrifuged at 3,000 rpm for 15 min. This was repeated three times, discarding the supernatant each time with the fourth extraction being poured through a Buchner filtering funnel. The acetone powder was allowed to air dry, followed by assay according to the procedures described

by McLord and Kilara, (1983). To a solution of crude polyphenols (100mg/10mL Citrate-Phosphate buffer, 0.02M, pH 5.5) 100mg of acetone powder (4,000  $\mu$ /mg protein) was added and allowed to stir for 30 min. with a stream of air bubbled through the slurry. The sample was centrifuged at 5,000 xg for 15 min. and the supernatant extracted 3X with 20 mL ethyl acetate. The ethyl acetate extracts were combined, taken to dryness by distillation under partial vacuum and 5mL water added, followed by lyophilization. The material was then assayed against Hela cells and the dose-response compared to crude polyphenol extracts that were not enzymically treated. The results (Figure 15M) showed a significant shift in the dose-response curve for the enzymically oxidized extract, showing that the oxidized products were more inhibitory than their native forms.

Example 9: Antioxidant Activity of Cocoa Extracts Containing Procyanidins

Evidence in the literature suggests a relationship between the consumption of naturally occurring antioxidants (Vitamins C, E and B-carotene) and a lowered incidence of disease, including cancer (Designing Foods, 1993; Caragay, 1992). It is generally thought that these antioxidants affect certain oxidative and free radical processes involved with some types of tumor promotion. Additionally, some plant polyphenolic compounds that have been shown to be anticarcinogenic, also possess substantial antioxidant activity (Ho et al., 1992; Huang et al., 1992).

To determine whether cocoa extracts containing procyanidins possessed antioxidant properties, a standard Rancimat method was employed. The procedures described in Examples 1, 2 and 3 were used to prepare cocoa extracts which were manipulated further to produce two fractions from gel permeation chromatography. These two fractions are actually combined fractions A through C, and D and E (See Figure 1) whose antioxidant properties



were compared against the synthetic antioxidants BHA and BHT.

Peanut Oil was pressed from unroasted peanuts after the skins were removed. Each test compound was  
 5 spiked into the oil at two levels, ~ 100 ppm and ~ 20 ppm, with the actual levels given in Table 7. 50  $\mu$ L of methanol solubilized antioxidant was added to each sample to aid in dispersion of the antioxidant. A control  
 10 sample was prepared with 50  $\mu$ L of methanol containing no antioxidant.

The samples were evaluated in duplicate, for oxidative stability using the Rancimat stability test at 100°C and 20 cc/min of air. Experimental parameters were  
 15 chosen to match those used with the Active Oxygen Method (AOM) or Swift Stability Test (Van Oosten et al., 1981). A typical Rancimat trace is shown in Figure 16. Results are reported in Table 8 as hours required to reach a peroxide level of 100 meq.

Table 7: Concentrations of Antioxidants

20	SAMPLE	LEVEL 1	LEVEL 2
			ppm
	Butylated Hydroxytoluene (BHT)	24	120
	Butylated Hydroxyanisole (BHA)	24	120
	Crude Ethyl Acetate Fraction of Cocoa	22	110
25	Fraction A-C	20	100
	Fraction D-E	20	100

Table 8: Oxidative Stability of Peanut Oil  
with Various Antioxidants

SAMPLE	20 ppm	100 ppm
	average	
Control	10.5 $\pm$ 0.7	
BHT	16.5 $\pm$ 2.1	12.5 $\pm$ 2.1
BHA	13.5 $\pm$ 2.1	14.0 $\pm$ 1.4
Crude Cocoa Fraction	18.0 $\pm$ 0.0	19.0 $\pm$ 1.4
Fraction A-C	16.0 $\pm$ 6.4	17.5 $\pm$ 0.0
Fraction D-E	14.0 $\pm$ 1.4	12.5 $\pm$ 0.7

These results demonstrated increased oxidative stability of peanut oil with all of the additives tested. The highest increase in oxidative stability was realized by the sample spiked with the crude ethyl acetate extract of cocoa. These results demonstrated that cocoa extracts containing procyanidins have antioxidant potential equal to or greater than equal amounts of synthetic BHA and BHT. Accordingly, the invention may be employed in place of BHT or BHA in known utilities of BHA or BHT, such as for instance as an antioxidant and/or food additive. And, in this regard, it is noted too that the invention is from an edible source. Given these results, the skilled artisan can also readily determine a suitable amount of the invention to employ in such "BHA or BHT" utilities, e.g., the quantity to add to food, without undue experimentation.

**Example 10: Topoisomerase II Inhibition Study**

DNA topoisomerase I and II are enzymes that catalyze the breaking and rejoining of DNA strands, thereby controlling the topological states of DNA (Wang, 1985). In addition to the study of the intracellular function of topoisomerase, one of the most significant findings has been the identification of topoisomerase II as the primary cellular target for a number of clinically

important antitumor compounds (Yamashita et al., 1990) which include intercalating agents (m-AMSA, Adriamycin® and ellipticine) as well as nonintercalating epipodophyllotoxins. Several lines of evidence indicate that some antitumor drugs have the common property of stabilizing the DNA - topoisomerase II complex ("cleavable complex") which upon exposure to denaturing agents results in the induction of DNA cleavage (Muller et al., 1989). It has been suggested that the cleavable complex formation by antitumor drugs produces bulky DNA adducts that can lead to cell death.

According to this attractive model, a specific new inducer of DNA topoisomerase II cleavable complex is useful as an anti-cancer, anti-tumor or antineoplastic agent. In an attempt to identify cytotoxic compounds with activities that target DNA, the cocoa procyanidins were screened for enhanced cytotoxic activity against several DNA - damage sensitive cell lines and enzyme assay with human topoisomerase II obtained from lymphoma.

A. Decatenation of Kinetoplast DNA by Topoisomerase II

The in vitro inhibition of topoisomerase II decatenation of kinetoplast DNA, as described by Muller et al. (1989), was performed as follows. Nuclear extracts containing topoisomerase II activity were prepared from human lymphoma by modifications of the methods of Miller et al. (1981) and Danks et al. (1988). One unit of purified enzyme was enough to decatenate 0.25 µg of kinetoplast DNA in 30 min. at 34°C. Kinetoplast DNA was obtained from the trypanosome *Crithidia fasciculata*. Each reaction was carried out in a 0.5 mL microcentrifuge tube containing 19.5 µL H<sub>2</sub>O, 2.5 µL 10X buffer (1X buffer contains 50 mM tris-HCl, pH 8.0, 120 mM KCl, 10mM MgCl<sub>2</sub>, 0.5 mM ATP, 0.5 mM dithiothreitol and 30 µg BSA/mL), 1 µL kinetoplast DNA (0.2 µg), and 1 µL DMSO-containing cocoa procyanidin test fractions at various

concentrations. This combination was mixed thoroughly and kept on ice. One unit of topoisomerase was added immediately before incubation in a waterbath at 34°C for 30 min.

5           Following incubation, the decatenation assay was stopped by the addition of 5  $\mu$ L stop buffer (5% sarkosyl, 0.0025% bromophenol blue, 25% glycerol) and placed on ice. DNA was electrophoresed on a 1% agarose gel in TAE buffer containing ethidium bromide (0.5  
10  $\mu$ g/mL). Ultraviolet illumination at 310 nm wavelength allowed the visualization of DNA. The gels were photographed using a Polaroid Land camera.

Figure 17 shows the results of these experiments. Fully catenated kinetoplast DNA does not  
15 migrate into a 1% agarose gel. Decatenation of kinetoplast DNA by topoisomerase II generates bands of monomeric DNA (monomer circle, forms I and II) which do migrate into the gel. Inhibition of the enzyme by addition of cocoa procyanidins is apparent by the  
20 progressive disappearance of the monomer bands as a function of increasing concentration. Based on these results, cocoa procyanidin fractions A, B, D, and E were shown to inhibit topoisomerase II at concentrations ranging from 0.5 to 5.0  $\mu$ g/mL. These inhibitor  
25 concentrations were very similar to those obtained for mitoxanthrone and m-AMSA [4'-(9-acridinylamino)methanesulfon-m-anisidide].

#### B. Drug Sensitive Cell Lines

Cocoa procyanidins were screened for  
30 cytotoxicity against several DNA-damage sensitive cell lines. One of the cell lines was the xrs-6 DNA double strand break repair mutant developed by P. Jeggo (Kemp et al., 1984). The DNA repair deficiency of the xrs-6 cell line renders them particularly sensitive to x-  
35 irradiation, to compounds that produce DNA double strand breaks directly, such as bleomycin, and to compounds that

inhibit topoisomerase II, and thus may indirectly induce double strand breaks as suggested by Warters et al.

(1991). The cytotoxicity toward the repair deficient line was compared to the cytotoxicity against a DNA

- 5 repair proficient CHO line, BR1. Enhanced cytotoxicity towards the repair deficient (xrs-6) line was interpreted as evidence for DNA cleavable double strand break formation.

- The DNA repair competent CHO line, BR1, was developed by Barrows et al. (1987) and expresses O<sup>6</sup>-alkylguanine - DNA - alkyltransferase in addition to normal CHO DNA repair enzymes. The CHO double strand break repair deficient line (xrs-6) was a generous gift from Dr. P. Jeggo and co-workers (Jeggo et al., 1989).  
15 Both of these lines were grown as monolayers in alpha-MEM containing serum and antibiotics as described in Example 6. Cells were maintained at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Before treatment with cocoa procyanidins, cells grown as monolayers were detached with trypsin treatment. Assays were performed using the  
20 MTT assay procedure described in Example 6.

- The results (Figure 18) indicated no enhanced cytotoxicity towards the xrs-6 cells suggesting that the cocoa procyanidins inhibited topoisomerase II in a manner  
25 different from cleavable double strand break formation. That is, the cocoa procyanidins interact with topoisomerase II before it has interacted with the DNA to form a noncleavable complex.

- Noncleavable complex forming compounds are relatively new discoveries. Members of the  
30 anthracyclines, podophyllin alkaloids, anthracenediones, acridines, and ellipticines are all approved for clinical anti-cancer, anti-tumor or antineoplastic use, and they produce cleavable complexes (Liu, 1989). Several new  
35 classes of topoisomerase II inhibitors have recently been identified which do not appear to produce cleavable

complexes. These include amonafide (Hsiang et al., 1989), distamycin (Fesen et al., 1989), flavanoids (Yamashita et al., 1990), saintopin (Yamashita et al., 1991), membranone (Drake et al., 1989), terpenoids (Kawada et al., 1991), anthracyclazines (Fry et al., 1985), dioxopiperazines (Tanabe et al., 1991), and the marine acridine - dercitin (Burres et al., 1989).

Since the cocoa procyanidins inactivate topoisomerase II before cleavable complexes are formed, they have chemotherapy value either alone or in combination with other known and mechanistically defined topoisomerase II inhibitors. Additionally, cocoa procyanidins also appear to be a novel class of topoisomerase II inhibitors, (Kashiwada et al., 1993) and may thus be less toxic to cells than other known inhibitors, thereby enhancing their utility in chemotherapy.

The human breast cancer cell line MCF-7 (ADR) which expresses a membrane bound glycoprotein (gp170) to confer multi-drug resistance (Leonessa et al., 1994) and its parental line MCF-7 p168 were used to assay the effects of cocoa fraction D & E. As shown in Figure 19, the parental line was inhibited at increasing dose levels of fraction D & E, whereas the Adriamycin (ADR) resistant line was less effected at the higher doses. These results show that cocoa fraction D & E has an effect on multi-drug resistant cell lines.

#### Example 11: Synthesis of Procyanidins

The synthesis of procyanidins was performed according to the procedures developed by Delcour et al. (1983), with modification. In addition to condensing (+)-catechin with dihydroquercetin under reducing conditions, (-)-epicatechin was also used to reflect the high concentrations of (-)-epicatechin that naturally occur in unfermented cocoa beans. The synthesis products were isolated, purified, analyzed, and identified by the

procedures described in Examples 3, 4 and 5. In this manner, the biflavanoids, triflavanoids and tetraflavanoids are prepared and used as analytical standards and, in the manner described above with respect to cocoa extracts.

Example 12: Assay of Normal Phase Semi-Preparative Fractions

Since the polyphenol extracts are compositionally complex, it was necessary to determine which components were active against cancer cell lines for further purification, dose-response assays and comprehensive structural identification. A normal phase semi preparative HPLC separation (Example 3B) was used to separate cocoa procyanidins on the basis of oligomeric size. In addition to the original extract, twelve fractions were prepared (Figures 2B and 15 O) and assayed at 100 $\mu$ g/mL and 25 $\mu$ g/mL doses against Hela to determine which oligomer possessed the greatest activity. As shown in Figure 20, fractions 4-11 (pentamer-dodecamer) demonstrated IC<sub>50</sub> values of approximately 25 $\mu$ g/mL. These results indicated that these specific oligomers had the greatest activity against Hela cells. Additionally, normal phase HPLC analysis of cocoa fraction D & E indicated that this fraction was enriched with these oligomers.

From the foregoing, it is clear that the extract and cocoa polyphenols, as well as the compositions method and kit, of the invention have utility. In this regard, it is mentioned that the invention is from an edible source and, that the activity in vitro can demonstrate at least some activity in vivo, especially considering the doses discussed above.

Additionally, the above description shows that the extract and cocoa polyphenols, as well as the compositions, method and kit have antioxidant activity like that of BHT and BHA, as well as oxidative stability.